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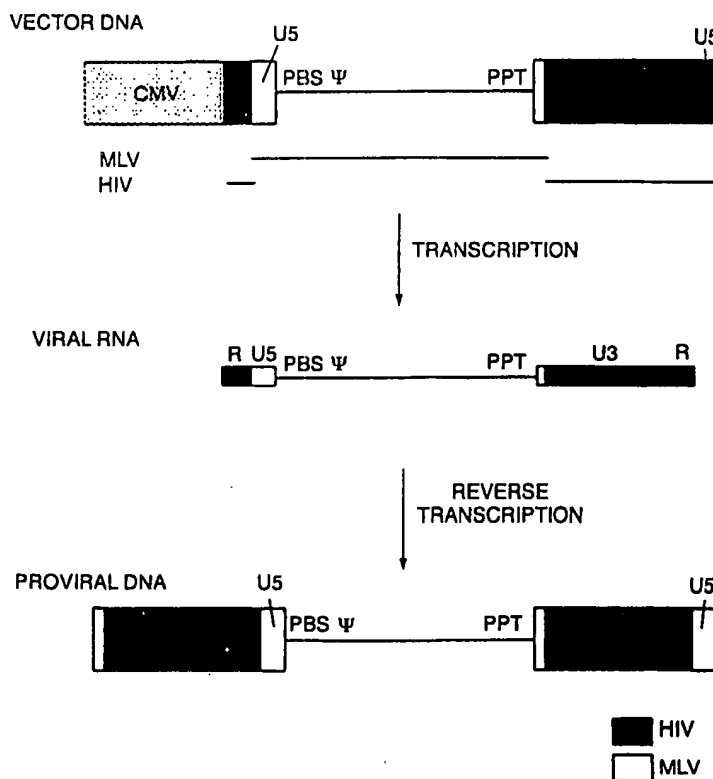
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(54) Title: RETROVIRAL VECTORS**(57) Abstract**

Retroviral vector particles having an RNA genome carrying sequences which provide in the DNA provirus at least one selected gene located within an intron in a transcription unit of the provirus, which transcription unit further comprises a polynucleotide response element responsive to a nucleus to cytoplasm transport factor such as HIV Rev. Expression of the selected genes is thus rendered Rev-dependent and so is dependent upon the presence of HIV.



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RETROVIRAL VECTORS

This invention relates to retroviral vector particles and to DNA constructs encoding RNA genomes for retroviral vectors. In particular it relates to retroviral vectors for gene therapy for treatment or prevention of retrovirus infections, such as HIV.

Therapeutic molecules for use in HIV gene therapy include ribozymes, trans-dominant proteins, scFv molecules, antisense constructs and TAR and RRE decoys (reviewed in Yu *et al.* 1994). These molecules are envisaged to act both as therapy against already infected cells and as protective 'intracellular immunisation' (Baltimore, 1988) in uninfected cells. In addition, the use of toxins (suicide genes) or immunological markers has also been proposed as a means of killing infected cells and so reducing the viral load in the patient.

For many of these molecules, it is desirable that expression can be regulated. This is clearly the case for suicide genes, but the constitutive expression of a therapeutic protein may also be undesirable in that it may cause some cellular toxicity or lead to a host immunological response. Furthermore, toxic side effects are also possible from the expression of RNA molecules in cells, including the inappropriate induction of interferon responses. In the case of HIV, the viral LTR promoter itself has the characteristic of being an inducible promoter, directing low basal levels of transcription in the absence of the virally encoded Tat trans-activator protein (Arya *et al.* 1985). Tat-inducibility is a property of sequences in the U3 region of the LTR and the TAR sequence in the R region (Berkhout and Jeang, 1992). Allowing the HIV LTR to direct expression of a therapeutic gene will therefore limit its expression to those cells infected by HIV. Several groups have already started exploring ways of using this property of the LTR and have demonstrated inducible expression of genes upon HIV infection (Caruso *et al.* 1992, Brady *et al.*

1994). Recently we have developed a novel vector system, the TIN system, that has clear advantages for HIV gene therapy (PCT/GB96/01230). This system comprises novel MLV based Tat-inducible (TIN) vectors, designed for the 5' positioning of a Tat induced therapeutic gene (TITG), and incorporating features to allow the efficient packaging, reverse transcription and integration of the vector genome by the MLV machinery. To facilitate the production of high levels of vector genomes in the producer cells (standard MLV packaging lines or our transient transfection system (Sonèoka *et al.* 1995)), the packagable vector transcript is preferably driven from the CMV promoter rather than the natural vector MLV LTR. A clear advantage of this system is the achievement of HIV control of gene expression from a simple transcription unit which can be transduced by the standard MLV based vector technology. The TIN vector system was proposed to have additional advantages in that the low basal levels of transcription from the HIV LTR promoter would reduce problems of interference with any downstream constitutive promoters, facilitating stable expression of any constitutive gene from the vector in the transduced cells.

Where the therapeutic gene is a non natural protein there always exists the possibility of immune recognition and destruction of the cell expressing this protein. This is clearly undesirable if the aim is to create a reservoir of HIV resistant cells in the patient. It is therefore important to have basal levels of expression as close to zero as possible. The data using TIN vectors that we have previously described clearly demonstrate very low basal levels of expression but the levels were still detectable. Furthermore it is known that in some target cells additional cellular factors may be present that enhance the basal transcription from the HIV promoter in the absence of Tat. Indeed some transcription from the HIV LTR must occur to produce Tat itself. In the T cell population exposure to cytokines, antigens and various stress factors such as hypoxia

or UV irradiation are all known to stimulate transcription from the HIV LTR (Valerie *et al* 1988; Nabel & Baltimore 1987). Given that these types of stimuli are characteristic features of immune responses there therefore exists the possibility that basal expression from TIN vectors could be elevated under certain conditions within an HIV infected patient. It is therefore desirable to provide additional controls on the basal levels of expression.

There is an additional regulatory circuit in HIV gene expression which involves the virally encoded Rev protein and its target RNA sequence the Rev response element (RRE) (reviewed by Cullen 1995 and explored by Naldini *et al* 1996). HIV has two introns in its genome and regulates splicing to generate unspliced RNA genomes and two classes of sub-genomic mRNA. To ensure that some RNA remains unspliced and is exported to the cytoplasm for incorporation into new virus particles the second intron contains a specific recognition sequence for a virally encoded protein called Rev. This RNA sequence is the Rev response element (RRE), for which a minimal functional sequence of 270 nucleotides has been identified (Huang *et al* 1991). Rev is a 13kD protein that specifically binds to RRE and activates the export of RRE-containing RNA (Malim *et al* 1989b). It appears that the HIV introns are recognised by the cellular splice commitment factors; but the splicing process is inefficient which leads to entrapment of the RNAs within the nucleus. The Rev/RRE interaction bypasses this entrapment and exports the RNA to the cytoplasm. If splicing is made more efficient then the RNA export becomes Rev independent (Chang and Sharp 1989; Hammariskjold *et al* 1994). If however gene expression is made more efficient then unspliced RNA can appear in the cytoplasm (e.g. D'Agostino *et al* 1992). In addition certain sequence elements referred to as cis inhibitory sequences (CRSs) might contribute to the Rev dependency of HIV by promoting nuclear entrapment and/or RNA instability (e.g. Cochrane *et al* 1991). There is therefore an

interaction between splicing efficiency, expression efficiency and CRSs that may influence Rev dependency. The interaction is not necessarily predictable or definable and may vary in different cell types (as discussed by Cullen 1995). The mechanistic details of Rev-mediated export have not
5 yet been established but cellular export proteins are involved (e.g. Fritz *et al* 1995).

One potential approach to achieving additional regulation of a retroviral vector for HIV gene therapy would therefore be to incorporate RRE into the vector rendering expression dependent upon Rev. Such an
10 approach may be obvious for vectors specifically based upon HIV itself as these will of necessity contain the Rev/RRE system, unless removed. For example Naldini *et al* (1996) use a fragment of HIV genome encompassing the normal major splice donor sequence, RRE and the normal splice acceptor sequence in the normal configuration in their transducing vector
15 pHR'. RRE is therefore contained within the HIV env intron. This fragment is presumed and is likely, but not proven, to render the expression of the genome responsive to Rev. In the vector described in this paper the coding sequence lies outside of the RRE containing intron.

A non-lentiviral retroviral vector incorporating the Rev/RRE
20 system has been described by Lisiewicz (US patent publication PB92-139336) whereby the RRE element is inserted into a retroviral vector or into the intron of a foreign gene contained within that vector. The constructions outlined make no reference to the presence of the splice donor sequence within the MLV vector, nor take into account any
25 requirement for inefficient splicing to achieve Rev function, and there is no description of precisely where to insert the RRE. The disclosure therefore suggests that in constructing a retroviral vector whose expression is dependent upon Rev the nature and location of the RRE is not material and the nature and location of additional introns or splice sequences is not
30 material.

We now show that a TIN vector can be converted into a Rev responsive vector by the insertion of RRE to create TRIN (Tat and Rev inducible) vectors. However, contrary to the finding in Lisiewicz, we demonstrate that simply inserting RRE into a TIN vector does not achieve
5 a strict dependency on Rev and that there is therefore no significant advantage in this construction with respect to reducing basal gene expression beyond that obtained with TIN vectors. Moreover, we show that the inclusion of an extended sequence derived from the HIV env region which contains RRE and the 3' splice acceptor sequence from env
10 produces a new vector pTRAC for which the basal transcription levels are undetectable in the absence of Tat and Rev. We propose that this undetectable expression is due to the combination of nuclear retention of the RNA via recognition of a hybrid MLV/HIV intron and the fact that any RNA that is spliced rather than exported via the Rev system will have the
15 reporter/therapeutic gene removed during splicing.

The invention therefore provides in one aspect a retroviral vector particle comprising a packagable RNA genome capable of being inserted into a target cell genome when in the form of a DNA provirus, said RNA genome carrying sequences which provide in the DNA provirus at
20 least one selected gene capable of being expressed in the target cell and located within an intron in a transcription unit of the provirus, which transcription unit further comprises a polynucleotide response element responsive to a nucleus to cytoplasm transport factor.

In another aspect, the invention provides a DNA construct
25 encoding the packagable RNA genome for the retroviral vector particle described herein, under the control of a promoter. The selected gene or genes may be present in or absent from the DNA construct. If they are absent, the DNA construct has an insertion site e.g. a unique restriction enzyme site at which the selected gene or genes may be inserted, the site

located within the intron such that it is flanked by a splice donor and a splice acceptor sequence.

In a further aspect, the invention provides a retroviral vector particle production system comprising a host cell transfected with a DNA
5 construct as described herein, said system capable of producing retroviral vector particles as described herein. The host cell may be a packaging cell line or it may be a suitable host cell transfected with nucleic acid sequences, present e.g. on plasmids, encoding the structural elements of the retroviral particles. The transfected host cell is also referred to as a
10 producer cell.

In yet another aspect, the invention provides a retroviral vector particle production system comprising a set of nucleic acid sequences encoding the components of a retroviral vector particle as described herein.

15 In a still further aspect, the invention provides the use of retroviral vector particles as described herein for gene therapy, and infected or transduced target cells resulting from such use.

The retroviral vector particle according to the invention therefore provides a means for inserting into a target cell a therapeutic
20 gene whose expression in the target cell is dependent upon the presence of a factor which enables the transport of an RNA transcript containing the transcribed gene, into the cytoplasm.

The response element in the retroviral vector genome is chosen according to the specific conditions under which expression of the
25 therapeutic gene is desired. If expression is to be HIV dependent, then a suitable response element is RRE or a functional equivalent which responds to Rev. A functional equivalent of RRE may be for example a portion of RRE or a mutated or otherwise manipulated version of RRE which retains the desired activity.

The transport factor may thus be HIV Rev, rendering expression of the therapeutic gene dependent upon the presence of HIV. The transport factor may alternatively be any factor, originating e.g. from other viruses or from host cells, which is analogous to Rev in that it
5 enables transport to the cytoplasm via a specific interaction with the response element. Systems analogous to the Rev/RRE system are known, for example in other retroviruses. One such example is the rex/RxRE system in HTLV-1.

In order that RNA transcripts containing the transcribed
10 therapeutic gene may be transported to the cytoplasm in the presence of the appropriate transport factor, the intron containing the therapeutic gene needs to be an inefficiently spliced intron. This may be achieved in a variety of ways. The preferred method described herein for achieving a Rev-dependent intron in an MLV-based vector is to use the MLV splice
15 donor site and the HIV envelope gene 3' splice site. It is within the ability of those skilled in the art to devise other suitable splice site or intron sequence combinations for HIV Rev-dependency.

Preferably, the RRE or other response element is located within the intron, to ensure Rev- (or other transport factor) dependent
20 expression. Conveniently, RRE and the 3' splice acceptor site may be provided in a sequence derived from the HIV env region.

Advantageously, the genome of the retroviral vector particle according to the invention will have its packaging site located within the intron containing the therapeutic gene. This means that RNA that is
25 unspliced will be trapped in the nucleus and therefore unavailable for packaging, and RNA that is spliced will have the packaging site deleted and will also be unavailable for packaging. This feature contributes to the safety of the vector system, and is present in the preferred vector configuration described herein.

The retroviral vector particle may be MLV-based. MLV systems are so far the most widely used retroviral vector systems and have been used in human gene therapy applications. Other retroviruses may be used instead however, including other oncoretroviruses (the sub-group of retroviruses containing MLV), and lentiviruses (the sub-group of retroviruses containing HIV). Examples include ASLV, SNV and RSV all of which have been split into packaging and vector components for retroviral vector particle production systems. The retroviral vector particle according to the invention may be based on a genetically or otherwise (e.g. by specific choice of packaging cell system) altered version of a particular retrovirus.

That the vector particle according to the invention is "based on" a particular retrovirus means that the vector is derived from that particular retrovirus. The genome of the vector particle comprises components from that retrovirus as a backbone. The vector particle contains essential vector components compatible with the RNA genome, including reverse transcription and integration systems. Usually these will include gag and pol proteins derived from the particular retrovirus. Thus, the majority of the structural components of the vector particle will normally be derived from that retrovirus, although they may have been altered genetically or otherwise so as to provide desired useful properties. However, certain structural components and in particular the env proteins, may originate from a different virus. The vector host range and cell types infected or transduced can be altered by using different env genes in the vector particle production system to give the vector particle a different specificity.

Preferably, the retrovirus vector genome contains the minimum retroviral material necessary to function. This is important from a safety aspect; possible reconstruction of infectious virus particles must be avoided. In any case, the retroviral vector will be replication defective.

Also, by avoiding expression of unwanted virus proteins in the target cell, undesirable immune responses are reduced. Gag-pol and env are therefore supplied in trans in the vector particle production system.

However the retrovirus vector genome clearly needs to
5 contain the elements required for obtaining sufficiently high viral titres of vector particles from the producer cell, and for allowing it to integrate into the target cell genome. It will be evident that in order to function as a vector the retroviral vector particle according to the invention will need to have a reverse transcription system (compatible reverse transcription and
10 primer binding sites) and an integration system (compatible integrase and integration sites) allowing conversion to the provirus and integration of the double-stranded DNA into the host cell genome. Additionally, the vector genome will need to contain a packaging signal.

In a vector particle according to the invention which is
15 designed for use in gene therapy against a particular retrovirus, there is preferably also a control at the transcriptional level which requires the presence of that retrovirus in order to initiate or upregulate transcription of the therapeutic gene or genes. For anti-HIV gene therapy, the vector genome is preferably constructed such that in the DNA provirus the 5' LTR
20 comprises HIV sequences sufficient to render the therapeutic gene Tat-inducible, and the therapeutic gene is located between the LTRs, yet the vector is based on a simple retroviral vector such as MLV. The therapeutic gene is under the transcriptional control of the promoter in the 5' LTR but not otherwise operably linked to any other promoter from the vector
25 genome. This is the principle behind the TIN vectors discussed above.

In more detail, TIN vectors are constructed with the following points in mind. Tat acts on the TAR region of R, but also requires sequences in the U3 region to function properly. Certain sections of both U3 and R can be removed while still leaving an effective Tat-responsive
30 element. Deletions in HIV-2 U3 result in promoters with lower basal levels

of transcription that still remain responsive to Tat (Brady *et al* 1990). Thus, the transcriptional control is preferably provided by an HIV Tat-inducible promoter comprising the functional portions of both U3 and R from HIV. Alternatively certain U3 and/or R sequences from other retroviruses, which
5 are responsive to the HIV Tat protein, may be used, for example U3 from RSV linked to R from HIV. The TIN vector provides a means of preserving the Tat response within the context of a simple retroviral vector e.g. one based on MLV such that MLV packaging systems may be used.

The selected gene located in the intron of the vector genome
10 according to the invention is preferably a therapeutic gene, that is it encodes a gene product which is active against infection or disease. Where the vector particle is for use in anti-HIV gene therapy, the product of the therapeutic gene will have an appropriate activity for that purpose. Therapeutic genes may encode for example an anti-sense RNA, a
15 ribozyme, a transdominant negative mutant of a target protein, a toxin, a conditional toxin, an antigen that induces antibodies or helper T-cells or cytotoxic T-cells, a single chain antibody or a tumour suppressor protein.

Where two or more genes are present in the same transcription unit, there may be an internal ribosome entry site (IRES) e.g.
20 from picornaviral RNA, to allow both genes to be translated from a single transcript. Retroviruses incorporating IRES sequences have been constructed by others.

It will be evident that the term "gene" is used loosely here, and includes any nucleic acid coding for the desired polypeptide. Usually,
25 the genes delivered by the vector particle according to the invention will be cDNAs.

A further gene may also be present outside the intron containing the therapeutic gene and under the control of a separate promoter. This further gene may encode for example a selectable marker,
30 or a further therapeutic agent which may be among the therapeutic agents

listed above. Expression of this gene may be constitutive; in the case of a selectable marker this may be useful for selecting successfully transfected packaging cells, or packaging cells which are producing particularly high titers of the retroviral vector. Alternatively or additionally, the selectable
5 marker may be useful for selecting cells which have been successfully infected with the retroviral vector and have the provirus integrated into their own genome.

One way of performing gene therapy is to extract cells from a patient, infect the extracted cells with a retroviral vector and reintroduce the
10 cells back into the patient. A selectable marker may be used to provide a means for enriching for infected or transduced cells or positively selecting for only those cells which have been infected or transduced, before reintroducing the cells into the patient. This procedure may increase the chances of success of the therapy. Selectable markers may be for
15 instance drug resistance genes, metabolic enzyme genes, or any other selectable markers known in the art.

However, it will be evident that for many gene therapy applications of retroviral vectors, selection for expression of a marker gene may not be possible or necessary. Indeed expression of a selection marker,
20 while convenient for *in vitro* studies, could be deleterious *in vivo* because of the inappropriate induction of cytotoxic T lymphocytes (CTLs) directed against the foreign marker protein. Also, it is possible that for *in vivo* applications, vectors without any internal promoters will be preferable. The presence of internal promoters can affect for example the transduction titres
25 obtainable from a packaging cell line and the stability of the integrated vector.

The DNA construct according to the invention which encodes the packagable RNA genome preferably comprises a promoter originating from a source other than the first or second retrovirus. Particularly
30 preferred are strong promoters such as the CMV promoter which give rise

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to a high level of expression of the vector RNA in the producer cell line.

The invention will now be further described with reference to the accompanying drawings in which:

Figure 1 shows a schematic outline of constructs according to the invention. The vector genome in the producer cell is shown (i). In the producer cell Rev is provided either by cotransfection or in a stable cell line by procedures known to those ordinarily skilled. The resulting vector derived RNA (ii) is unspliced and is exported to the cytoplasm by the Rev protein. In the target cell the vector genome is as in (i) but the only RNA that appears in the cytoplasm has the structure shown in (iii) because Rev is absent. Upon HIV infection when Rev is produced by the infecting virus then the retroviral vector derived RNA that reaches the cytoplasm will have the structure shown in (iv) i.e. identical to that in the producer cell (ii);

Figure 2 shows vectors used in the construction of a universal TIN vector (pTIN511);

Figure 3 shows construction of an RRE splice acceptor cassette (RAC);

Figure 4 shows construction of pTRAC;

Figure 5 shows construction of pTRAC-TG;

Figure 6 shows construction of pTRIN-TG; and

Figure 7 shows the prototype TIN vector pTIN414.

Figure 8 shows the principle of TIN vectors. The specific example of an MLV-based Tat-inducible vector is given. The packagable RNA genome comprises from the 5'end at least the functional part of the R region of HIV, all or a functional part of the MLV U5 region, a functional MLV primer binding site for first strand reverse transcription, a functional MLV packaging site, an insertion site for insertion of one or more therapeutic genes (into the DNA copy), a functional MLV primer binding site for second strand reverse transcription, a short (e.g. 10 - 100 nucleotides) sequence recognised by the MLV integration system, all or a

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substantial part of the HIV U3 region, and an R region corresponding to the R region at the 5'end. Vector and proviral DNA are also shown.

Preparation of the constructs is described in detail in the Examples which follow.

5 This is the first description of the incorporation of an extended HIV-1 RRE element combined with an HIV-1 splice acceptor site into an MLV vector. It is the first description of a retroviral vector where the therapeutic gene is contained within an intron. The invention is particularly useful when combined with a TIN vector. That vector could be a single
10 transcription unit vector or could contain a second transcription unit as described for pTIN501. The retroviral vector could be based on any retrovirus including lenti-viruses. In the case of lentiviral vectors a vector has been described that contains the HIV-1 RRE and the major splice site but the therapeutic gene is not included within an intron and the Rev/RRE
15 system is not used to manipulate the expression of the therapeutic gene (Naldini *et al* 1996). The components of the intron in the present invention are preferably derived from MLV and HIV.

COMPONENTS OF MLV BASED TAT-INDUCIBLE (TIN) VECTORS

20 We have constructed a series of vectors that are packagable by standard MLV components, can be reverse transcribed and integrated by the MLV machinery following infection or transduction of a cell, and will allow Tat-inducible expression of a therapeutic gene from the transduced vector. The basic components of such a vector are as follows (Figure 8):

25

1. **Tat-inducibility.** The Tat-inducibility of the HIV LTR promoter is a property of sequences in the U3 region of the promoter and also the TAR element in R (Berkhout and Jeang, 1992). In addition, some other promoters, including the U3 regions from HIV-2 and RSV can substitute for
30 the HIV-1 U3 regions to allow Tat transactivation (Liu *et al.* 1994). In order

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for the U3 element to appear in the 5' LTR following reverse transcription, it must be present in the 3' LTR of the viral RNA. The vector therefore contains the HIV U3 and R sequences at the 5'LTR.

5 **2. Reverse transcription.** MLV RT initiates reverse transcription at the primer binding site (PBS). This initial (-) strand synthesis extends into U5 and R sequences, forming the first 'strong stop' DNA strand. The RNaseH moiety of RT then degrades the RNA in this hybrid, allowing the exposed DNA to hybridise with the homologous R region in the 3' LTR of the
10 provirus. The homology between the 5' and 3' R regions enables the polymerase to switch strands and continue synthesis along the (-) strand from the 3'LTR. (+) strand DNA synthesis is primed by the selective retention of an RNA fragment at the polypurine tract after RNase
15 degradation of the genomic RNA strand (reviewed in Katz and Skalka, 1994). The minimum requirements for MLV pol directed reverse transcription contained in the vector are therefore the PBS to initiate (-) strand DNA synthesis, the PPT to initiate (+) strand DNA synthesis and identical 5' and 3' R sequences to allow the first template switch. The
20 requirement for identical R sequences is met by having HIV R sequences in both 5' and 3' LTRs. In addition, as there is evidence to suggest that secondary structures in the 5' U5 region are also important for the initiation of reverse transcription (Cobrink *et al.* 1991), we have kept the MLV U5 sequences in the 5'LTR. The 3' U5 sequences do not appear in the
25 genomic RNA transcript; however, to ensure correct termination at the 3' R/U5 border during genomic transcription, we will use the HIV U5 region in the 3' LTR.

30 **3. Integration.** The termini of the reverse-transcribed molecule contain short, sometimes imperfect, inverted repeats of 2-23 bp, which the retroviral integrase recognises (reviewed in Katz and Skalka, 1994). For

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MLV, it has been demonstrated that only 9 bases at the end of a linear model substrate are sufficient for almost wild-type levels of integration in an in vitro integration assay system (Bushman and Craigie, 1990). These sequences are derived from the ends of the 3'U3 region and the 5'U5 region in the vector. This requirement is met by the vector containing 36 bases of MLV sequence at the 5' end of the 3' U3 and the whole of the MLV 5' U5 region.

4. Packaging components. Efficient packaging of a vector genome into a retroviral particle is dependent on a number of cis-acting sequences (reviewed in Linial and Miller, 1990). The most important sequence is the packaging signal, a highly structured region of RNA at the 5' region of the genome. In addition, other regions of the genome have been found to increase the efficiency of packaging, including sequences in gag p15. This region is included in standard MLV retroviral vectors, such as LXS_N (Miller and Rosman, 1989) and is also preserved in our constructs.

5. High titer retrovirus stocks following transient transfection. We have recently devised a system for the rapid production of high titer retroviral vectors (10^7 /ml) by transient transfection (Soneoka *et al.* 1995). A key feature of this system is that the powerful CMV promoter drives high level expression of the vector RNA in the producer cell line and is positioned so that the transcription start site of the vector RNA is exactly the same as the normal LTR-directed start site. To achieve this, we have placed the CMV promoter up to its transcriptional start site adjacent to the start of the HIV R region in the 5' LTR. This principle could be applied to other heterologous promoters, as long as the integrity of the retroviral transcription unit is maintained.

This system has been described for hybrid MLV-HIV vectors, but the same principle can be applied to other combinations of

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retroviruses, where one retrovirus is donating the cis-acting sequences required by its own packaging components provided in trans (e.g. SNV, RSV, ASLV etc.) and the other retrovirus is used because of the property of conditional expression of its LTR promoter. Further examples of such retroviral promoters are the HTLV-1 promoter (dependent on Tax protein) and the steroid-hormone inducible MMTV LTR (reviewed in Majors, 1990). The use of HTLV-1 LTR, for example, to direct expression of a suicide gene could find applications as a treatment for adult T-cell leukemia.

10 **ADVANTAGES OF THE VECTORS DESCRIBED HEREIN**

1. Reduced basal levels of expression of therapeutic genes leading to reduced toxicity and immune recognition of target cells.
2. Stricter dependency upon HIV infection to activate gene expression
- 15 3. Reduced availability of RNA carrying the packaging site leading to lower risk of inadvertent packaging of the RNA in the target cell.

EXAMPLES

20 **1. Construction of a universal TIN vector (pTIN511) (Figure 2)**

The starting molecule is pTIN500 (described in PCT/GB96/01230). This was derived from pTIN414 described in PCT/GB96/01230 (and described herein in detail below and illustrated in Figure 7). pTIN414 is the TIN vector equivalent of retroviral vector PHIT111 (Soneoka *et al* 1995), a derivative of LZSN (Adam *et al* 1991). Subsequent TIN vectors were derived from pTIN414 by replacement of internal sequences between unique *Spe*I and *Nhe*I sites. The *Spe*I site is located within the non-translated gag coding region upstream of the lacZ gene and the *Nhe*I site is in the 3' U3 region at the junction of the MLV and HIV-1 sequences. Plasmid pTIN500 contains the *Spe*I - *Nhe*I internal

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fragment from pBABEpuro. The SV-Puro cassette is deleted by digestion with *AccI* and religation. This produces pTIN510. A poly-linker is inserted into the unique *EcoRI* site in pTIN510.

5 Polylinker sequence (SEQ ID NO: 1)

EcoRI Sall XhoI BglII EcoRI
5'AATTCGTCGACCTCGAGATCCG;
GCAGCTGGAGCTCTAGGCAATT 5'

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This creates pTIN511 which has unique Sall, XhoI and BglII sites for the insertion of additional sequences.

The removal of the SV-Puro cassette is not critical for the current invention but serves to simplify the structure of the vector. There may be situations, obvious to one skilled in the area, when the retention of this or any additional cassette might be desirable.

15

Detailed construction of TIN414.

The co-ordinates of the sequences derived from the CMV promoter, the MLV vector LZSN (Adam *et al.* 1991) and the HIV-1 proviral clone WI3 (Kim *et al.* 1989) are indicated. The molecule was created using standard recombinant DNA techniques and in addition, recombinant PCR to create exact junctions between the different parts of the molecule (Higuchi 1990). Specifically

25

(i) Construction of the 5' CMV driven LTR

Plasmid pPE611 (Braddock *et al.* 1989) contains the human CMV promoter (from -521 to +1) joined exactly to the start of the HIV-1 R region (co-ordinates +1 to +80). An *XbaI* - *BamHI* fragment from this plasmid was ligated into the cloning vector pBluescript (Stratagene) to give

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plasmid pRV404. A PCR amplification was performed using plasmid pLNSX as the template, using primers 5'-gcgagctagcttcgaatcgtggtctcgctgttccttg-3' and 5'-ggccgctagcgttcagaactcgtcagttccaccac-3'. The PCR product so generated was digested with NheI and ligated into pRV404 at its NheI site to give plasmid pRV405. Two oligonucleotides of sequence 5'-ttaagcctcaataaagcttgcttgagtgccttcac-3' and 5'-cggtgaagcactcaaggcaagcttattgaggc-3' were annealed together to create a short duplex containing single stranded regions at either end corresponding to the overhangs present on AflII and BstBI restriction fragments. This molecule was ligated into plasmid pRV405 cut with AflII and BstBI, to give plasmid pRV406.

(ii) Construction of the 3' LTR

A HindIII - XbaI fragment from pLNSX was ligated into the cloning vector pSP72 to give plasmid pRV400. Plasmid pBX+ contains a BamHI - XbaI fragment from WI3 containing the whole 3' LTR from the HIV-1 genome. PCR amplification was performed on pBX+ using primers 5'-ccgcgctagcgatctcttgatctgtgatctaccac-3' and 5'-gcgagggtaccgtcgactgctagagatttccacactgac-3'. The PCR product was digested with KpnI and NheI and ligated into plasmid pRV400 digested with KpnI and NheI, to give plasmid pRV401. The ClaI - KpnI fragment of pRV401 was ligated into pBluescript digested with ClaI and KpnI, to give plasmid pRV408.

(iii) Addition of internal sequences.

A SacII - SpeI fragment from pRV406 was ligated into plasmid pRV408 to create plasmid pRV412. The SpeI - NheI fragment from pRV412 was replaced with the SpeI - NheI fragment from LZSN to give vector pTIN414 (Figure 7).

2. Construction of the RRE splice acceptor cassette (RAC) Figure 3.

The starting molecule is pWI3 (Kim *et al* 1989). This contains a full length proviral clone of isolate HIV-1 IIIb clone HXB2 (Ratner *et al* 1985; Genbank accession no.K03455). Coordinates refer to nucleotide
5 positions in the proviral DNA starting with the first nucleotide of the 5' LTR as 1 and are given in brackets following the restriction site. To facilitate cloning steps a subclone is created that contains only the envelope region of HIV-1. To achieve this a Sall (5785) to BamHI (8473) fragment from WI3 is inserted into the polylinker of the cloning vector pSP46 (Promega) to
10 produce plasmid pPE531. The RRE splice acceptor region (RAC) is further subcloned as a BglII (7620) to EcoRI (site in the pSP46 polylinker) into the polylinker site of pSP71 to create pRAC.

3. Construction of pTRAC (Figure 4)

15 The BglII(7620) to BamHI (8473) fragment is inserted into the unique BglII site in pTIN511. In the correct orientation the upstream BglII site is preserved and the downstream site is a hybrid BglII/BamHI site which is non-functional for either enzyme. pTRAC therefore retains three unique cloning sites upstream of the RAC .

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4. Construction of pTRAC-TG (Figure 5)

A coding sequence of choice is inserted into one of the unique restriction sites in pTRAC. This sequence is a therapeutic gene or a reporter gene. The sequence is prepared with appropriate restriction at
25 the termini and has an ATG codon for translation initiation. In the present example the RevM10 sequence is amplified from plasmid pM10 (Malim *et al* 1989a) using PCR primers incorporating flanking BglII (upstream) and BamHI (downstream sites).

- 20 -

Primer sequences:

GGCAGATCTATGGCAGGAAGAAGCGG - 3' (SEQ ID NO: 2)

GGCGGATCCTTCTTTAGTTCCTGACTCC - 3' (SEQ ID NO: 3)

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The amplified product is digested with BglI and BamHI and the product is ligated into the unique BglII site of pTRAC. The vector genome is renamed according to the therapeutic gene in this example pTRAC-TG becomes pTRAC-RevM10.

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Insertion of the gene in the correct orientation preserves the upstream BglII site but the downstream site is destroyed by the formation of a BglII/BamHI hybrid site.

5. Construction of pTRIN and pTRIN-TG (Figure 6)

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To construct an RRE cassette, a 359 base pair fragment encompassing the minimal fully functional RRE is amplified from pRAC using primers that locate with respect to the HIV proviral sequence coordinates at nucleotide 7705 and 8067 (or from pPE351 sequences from 7707 to 8066). The upstream primer adds an EcoRI site followed by a BamHI site and the downstream primer adds an EcoRI site.

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Primer 1 (SEQ ID NO: 4) (lower case is HIV-1 sequence 7705 to 7725)

5'CCGCGAATTCGGATCCaggagtagcaccaccaaggc

Primer 2 (SEQ ID NO: 5) (lower case is HIV-1 sequence from 8067 to 8047)

25

5'CCGCGAATTctccaactagcattccaaggc

The amplified product is digested with EcoRI and is ligated into the EcoRI site of pTIN510 to produce pTRIN. This now has a unique BamHI site for the insertion of any additional sequences.

A therapeutic gene or reporter gene is inserted into the unique BamHI site of pTRIN. In the present example the RevM10 cassette described above is inserted. The vector is renamed according to the therapeutic gene in this example pTRIN-TG becomes pTRIN-RevM10.

5

6. Analysis of pTRAC-TG and pTRIN-TG

Retroviral vector stocks are produced either by transient transfection of 293T cells according to methods of Soneoka *et al* 1995 or by the creation of producer cell lines by standard methods e.g. (Cosset *et al* 1995). MLV packaging components are provided in trans on two plasmid components - a gag-pol expression plasmid (pHIT60, an MLV gag-pol expression plasmid described in Soneoka *et al* 1995) and an amphotropic envelope expression plasmid (pHIT456 derived from plasmid SV-A-MLV-env described in Page *et al* 1990 and essentially the same as the ecotropic expression construct pHIT123 described in Soneoka *et al* 1995). Rev is provided in trans from a CMV rev expression plasmid. The following vector genomes are used:-

- a) standard MLV vector (pHITIII; Soneoka *et al* 1995)
- b) pTRIN-RevM10
- 20 c) pTRAC-RevM10
- d) pTRIN-lacZ
- e) pTRAC-lacZ
- f) pTIN414 (as described in PCT/GB96/01230) and shown in Figure 7.

25

The following additional plasmids are used in the analysis:-

- g) pTAT (A CMV Tat expression plasmid as described by Braddock *et al* 1989)
- h) pHCMVsrev (A Rev expression plasmid as described by Benko *et al* 1990).

30



Virus stocks are used to infect HeLa-CD4 cells and human U937 cells. In some cases repeat transfections are done as described by Cannon *et al* (1996). In this procedure the target cells are exposed to fresh retroviral vector stocks every 48 hours. Forty-eight hours after the final transduction the transduced cell populations are infected with HIV-1. After 48 and 72 hours cells are harvested and protein extracts are prepared by standard procedures and assayed for the expression of the marker or therapeutic gene. In addition viral spread through the culture is assessed by determining reverse transcriptase activity in culture supernatants every three days.

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CLAIMS

- 5 1. A retroviral vector particle comprising a packagable RNA genome capable of being inserted into a target cell genome when in the form of a DNA provirus, said RNA genome carrying sequences which provide in the DNA provirus at least one selected gene capable of being expressed in the target cell and located within an intron in a transcription
10 unit of the provirus, which transcription unit further comprises a polynucleotide response element responsive to a nucleus to cytoplasm transport factor.
2. The retroviral vector particle according to claim 1, wherein the polynucleotide response element is responsive to a transactivating
15 retroviral nucleus to cytoplasm transport factor.
3. The retroviral vector particle according to claim 2, wherein the polynucleotide response element is responsive to HIV Rev or a functional equivalent thereof.
4. The retroviral vector particle according to any one of claims 1
20 to 3, wherein the polynucleotide response element is the Rev response element (RRE) or a function equivalent thereof.
5. The retroviral vector particle according to any one of claims 1 to 4, wherein a selected gene is a therapeutic gene.
6. The retroviral vector particle according to any one of claims 1
25 to 5, based on an oncoretrovirus.
7. The retroviral vector particle according to any one of claims 1 to 6, based on murine leukemia virus (MLV).
8. The retroviral vector particle according to any one of claims 1 to 7, wherein the 5' long terminal repeat (LTR) of the provirus comprises
30 HIV U5 and R regions or functional portions thereof having Tat inducible

promoter activity, in place of the 5' LTR promoter function of the retrovirus on which the vector particle is based.

9. The retroviral vector particle according to any one of claims 1 to 8, wherein the packaging signal is contained within the intron in which
5 the selected gene is located.

10. A DNA construct encoding the packagable RNA genome for the retroviral vector particle according to any one of claims 1 to 9, operably linked to a promoter.

11. The DNA construct according to claim 10, wherein the
10 promoter is a strong promoter such as the CMV promoter.

12. The DNA construct according to claim 10 or claim 11, wherein the selected gene is absent and the construct has an insertion site within the intron at which the selected gene or genes may be inserted.

13. A retroviral vector particle production system comprising a
15 host cell transfected with the DNA construct according to any one of claims 10 to 12, said system capable of producing retroviral vector particles according to any one of claims 1 to 8.

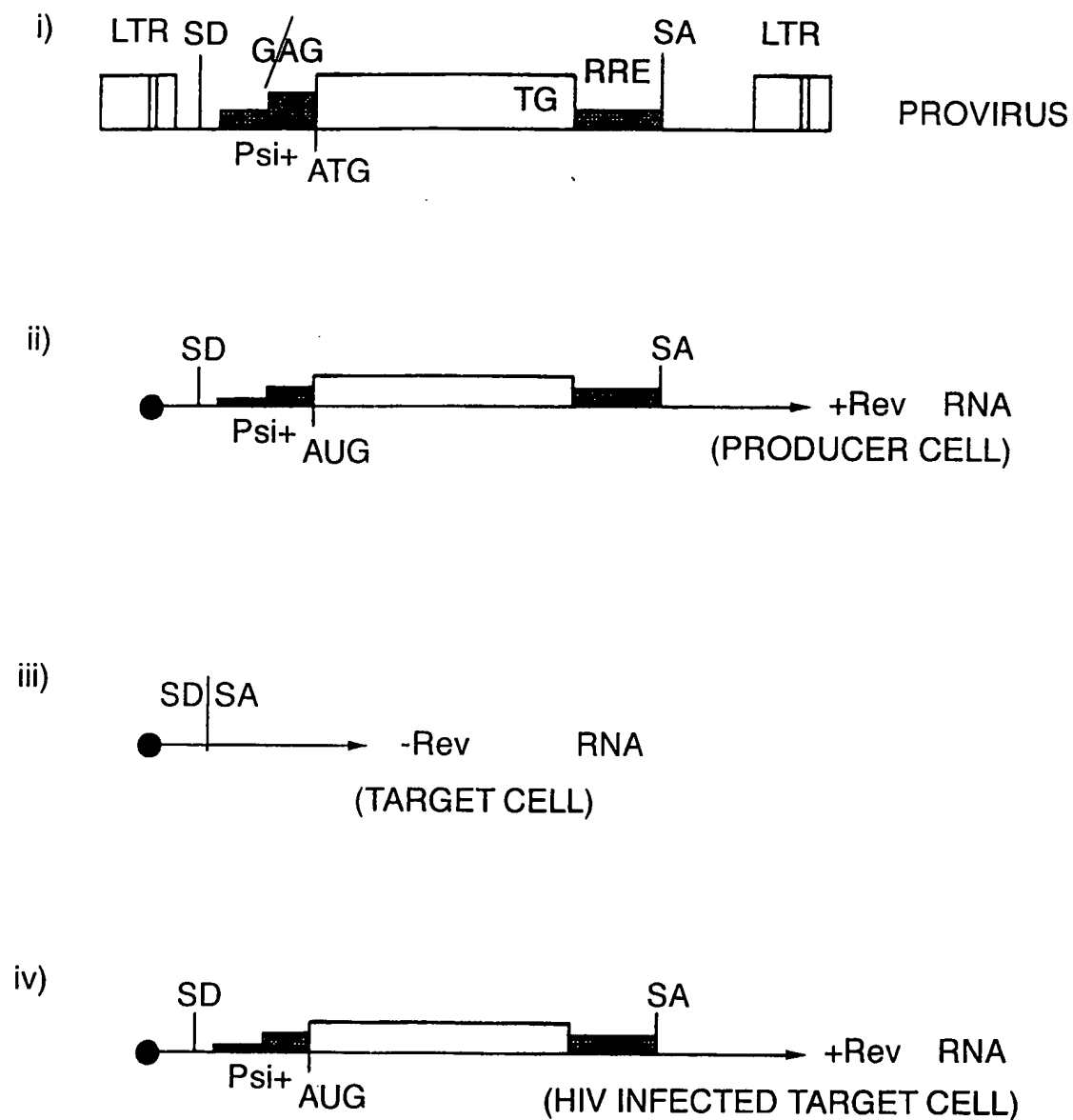
14. A retroviral vector particle production system comprising a set of nucleic acid sequences encoding the components of a retroviral vector
20 particle according to any one of claims 1 to 9.

15. The use of a retroviral vector according to any one of claims 1 to 9 for gene therapy for infection or transduction of a target cell.

16. Target cells resulting from the method according to claim 15.

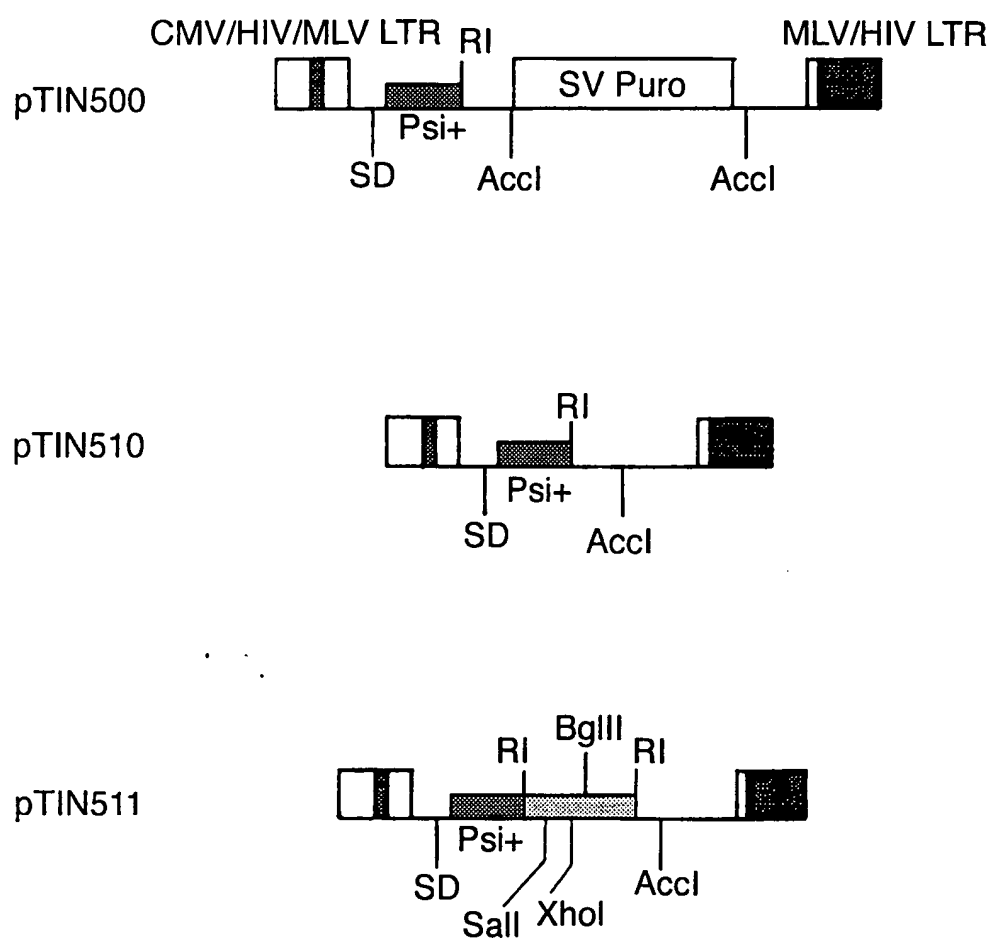
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Fig.1.



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Fig.2.



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Fig.3.

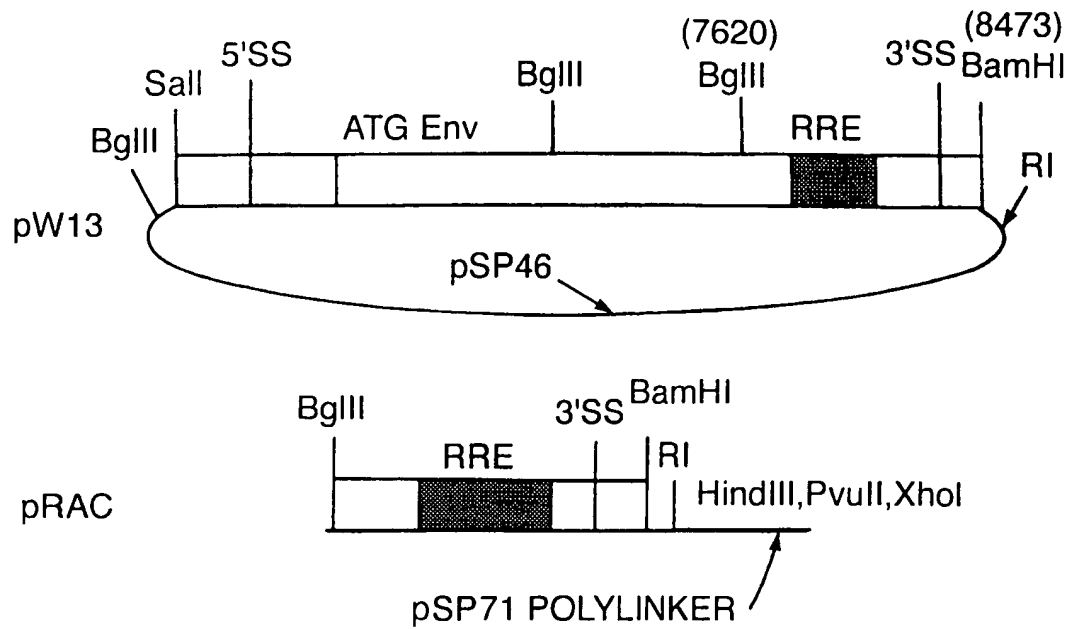


Fig.4.

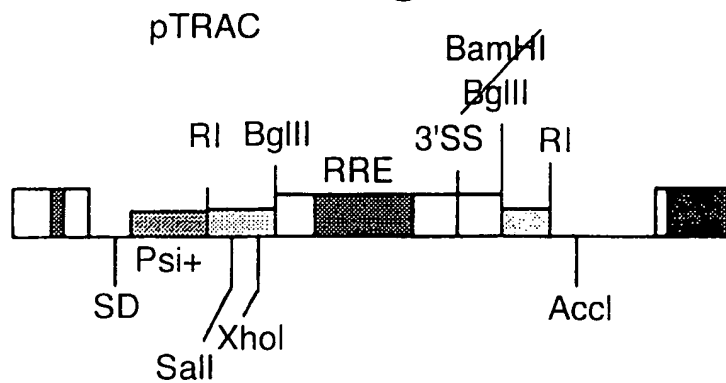
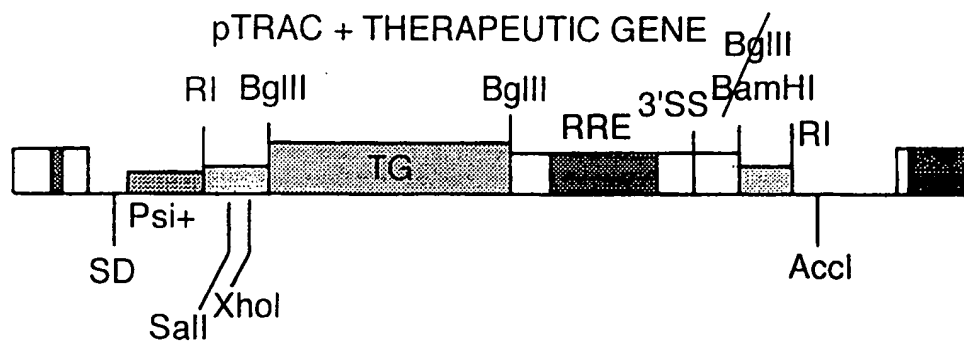


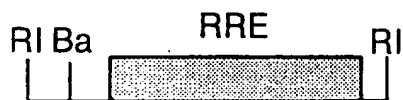
Fig.5.



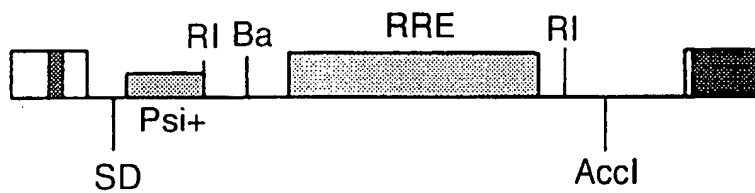
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Fig.6.

RRE CASSETTE



pTRIN



pTRIN-TG

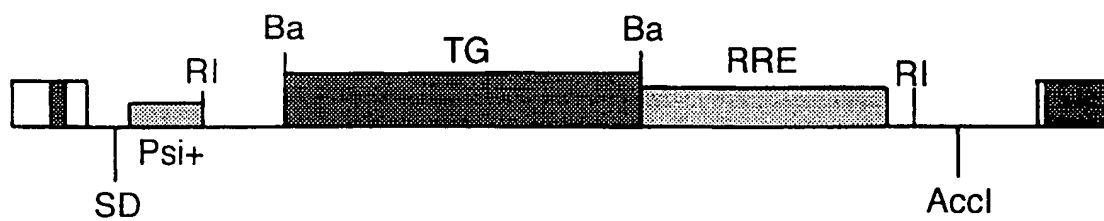
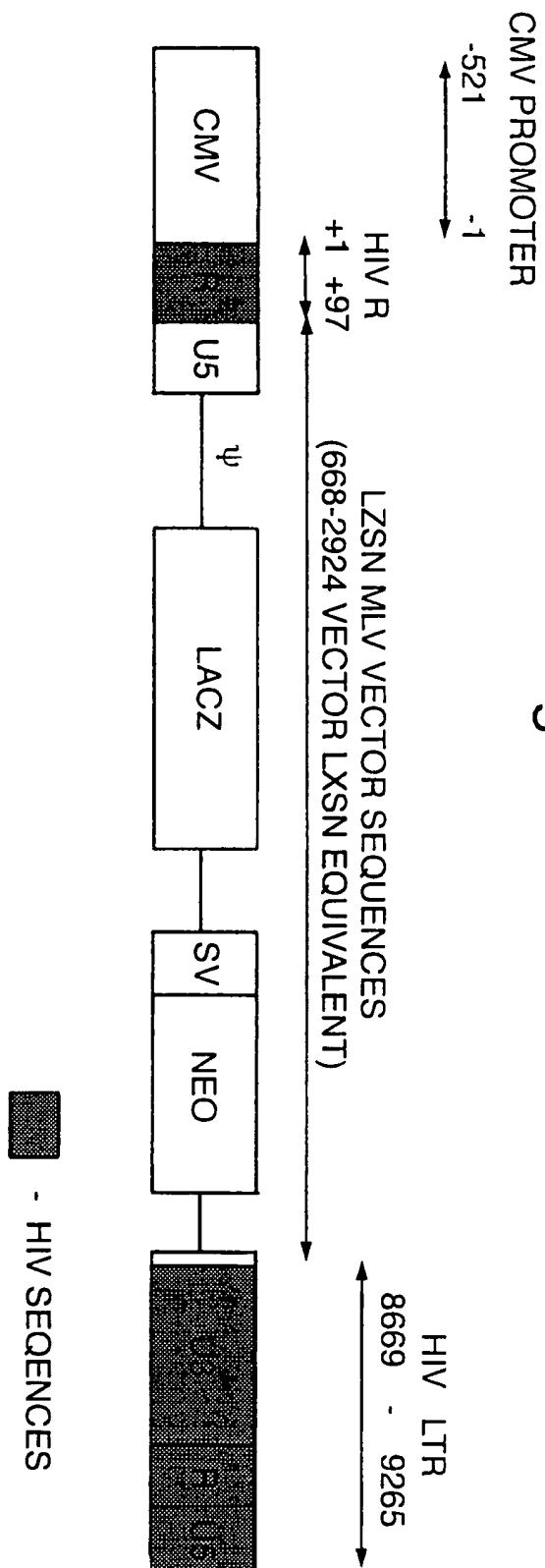
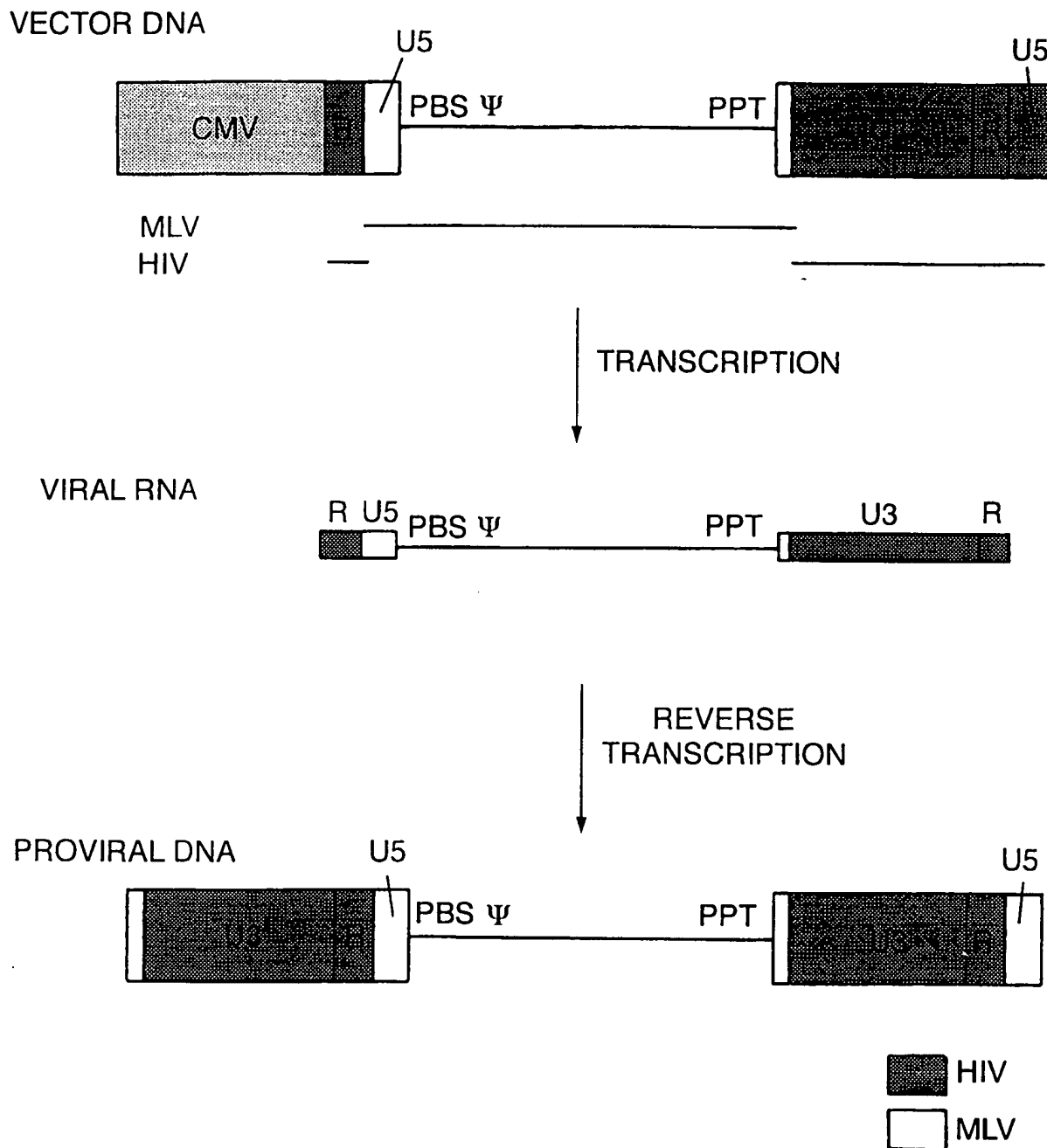


Fig. 7.



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Fig.8.



A. CLASSIFICATION OF SUBJECT MATTER IPC 6 C12N15/86 C12N5/10 C12N7/00 A61K48/00 - C07K14/16		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC 6 C12N C07K A61K		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 92 21750 A (US GOVERNMENT) 10 December 1992 see page 8, paragraph 5 - page 9, paragraph 3 see page 14 - page 16 see page 12, paragraph 2 - page 13, paragraph 3	1-16
Y	--- HOPE, T.J. ET AL.: "Steroid-receptor fusion of the human immunodeficiency virus type 1 Rev transactivator: mapping cryptic functions of the arginine-rich motif" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCE OF THE USA, vol. 87, October 1990, pages 7787-7791, XP002056927 see the whole document --- <div style="text-align: center;">-/-</div>	1-16
<div style="display: flex; justify-content: space-between;"> <input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input checked="" type="checkbox"/> Patent family members are listed in annex. </div>		
<div style="display: flex;"> <div style="flex: 1;"> <p>* Special categories of cited documents:</p> <p>*A* document defining the general state of the art which is not considered to be of particular relevance</p> <p>*E* earlier document but published on or after the international filing date</p> <p>*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>*O* document referring to an oral disclosure, use, exhibition or other means</p> <p>*P* document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="flex: 1;"> <p>*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>*&* document member of the same patent family</p> </div> </div>		
Date of the actual completion of the international search <div style="text-align: center; font-size: 1.2em;">26 February 1998</div>		Date of mailing of the international search report <div style="text-align: center; font-size: 1.2em;">17. 03. 98</div>
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016		Authorized officer <div style="text-align: center; font-size: 1.2em;">Smalt, R</div>

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MCLACHLIN J R ET AL: "RETROVIRAL-MEDIATED GENE TRANSFER" PROGRESS IN NUCLEIC ACID RESEARCH AND MOLECULAR BIOLOGY, vol. 38, 1990, pages 91-135, XP002030826 see page 119 ---	1-16
A	US 5 306 631 A (HARRISON GAIL ET AL) 26 April 1994 see claims 4,8,11; example 4 ---	1-16
A	WO 95 30755 A (HISAMITSU PHARMACEUTICAL CO ;SHIMADA TAKASHI (JP); AKIYAMA KATSUHI) 16 November 1995 see figure 1 & EP 0 759 471 A -----	11

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 97/02859

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
see FURTHER INFORMATION sheet PCT/ISA/210
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/GB 97/02859

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Remark : Although claim 15 is directed to a method of treatment of the human/animal body , the search has been carried out and based on the alleged effects of the compound/composition.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Intr

Application No

PCT/GB 97/02859

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9221750 A	10-12-92	AU 2007392 A CA 2109579 A EP 0588914 A JP 6510181 T	08-01-93 10-12-92 30-03-94 17-11-94
US 5306631 A	26-04-94	US 5554528 A US 5585254 A	10-09-96 17-12-96
WO 9530755 A	16-11-95	AU 2419595 A EP 0759471 A	29-11-95 26-02-97